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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



In re: Patent application of
Alan Gewirtz

Serial No.: 09/993,183

Filed: November 14, 2001

For: POST-TRANSCRIPTIONAL GENE SILENCING
BY RNAi IN MAMMALIAN CELLS

:
:
: Group Art Unit: 1635
:
:

: Examiner: Jon B. Ashen
:
:

: Conf. No. 6995
:
:

Declaration of Dr. Alan M. Gewirtz for Earlier Date of Invention under 37 C.F.R. §1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

*Considered
✓ D S
8/30/06*
I, Alan M. Gewirtz, declare that:

1. I am the sole inventor of the invention disclosed and claimed in U.S. Patent Application Serial No. 09/993,183, including all subject matter disclosed and claimed therein.
2. I am a Professor of Internal Medicine and Pathology-Laboratory Medicine at the University of Pennsylvania School of Medicine, Division of Hematology/Oncology. The Experiment set forth in the patent application, and all related experiments, were conducted in my laboratory and under my direction at the University of Pennsylvania in the United States of America.
3. Prior to August 3, 2000, Gokdan Demir, MD, working under my direction, began a series of experiments with me, on the project reported in US Patent Application 09/993,183, first filed as Provisional Patent Application 60/248,346, filed November 14, 2000. I initially summarized the concept of the invention on the page reproduced at Tab 1 attached hereto, with dates redacted. All dates are similarly redacted on the attached exhibits. No other change was

made to the document before copying and attaching the pages hereto. If the Examiner wishes to see the actual, dated documents, Applicant can comply, and has the entire notebook in which the pages were recorded in date-sequence order.

4. Initially, reliable protocols were optimized for isolating total RNA and for preparing double-stranded RNA (dsRNA). The human c-kit gene target has been well characterized in the literature, and previously used in other experiments in human cells in the Gewirtz laboratory. Throughout the course of these experiments, spectrophotometric analyses were conducted in the laboratory of Dr. Stephen G. Emerson at the University of Pennsylvania.

5. To begin, a portion of the 5' end of c-Kit genomic cDNA was subcloned into expression vector pcDNA3 (containing T7 and SP6 promoters) by digesting with BamHI. See Tab 2 attached hereto. Using the c-Kit gene as a target, the effect of dsRNA on the expression of the c-kit receptor (KitR) was evaluated in CHP 100 neuroepithelioma (human melanoma) cells and HL-60 (human leukemia) cells, both of which were known to express KitR. See Tabs 3 and 4 attached hereto. Cell lines were maintained in RPMI 1640 media (GibcoBRL, Gaithersburg, Md.) containing 10% BCS. Varying amounts (150-350 µg/ml) of Kit dsRNA (KdsRNA) were added to the culture media. See Tabs 3, 4 and 6-10 attached hereto.

6. As a control, GFP dsRNA (GdsRNA) was added. See Tabs 5 and 6 attached hereto. As a control, 724 bp of Green Fluorescent Protein (GFP) cDNA was subcloned into pcDNA3 by digesting with EcoRI and HindIII.

7. Subcloned vectors were amplified in a chemically competent strain of E. coli DH5_α cells. *In vitro* transcription reactions were carried out using known methods to linearize the plasmids using EcoRV and HindIII to synthesize the sense and corresponding antisense RNA strands, respectively. Digested plasmids were treated with Proteinase K to inactive any RNases, and purified as a template for transcription by commercial methods. RNA polymerases were from Promega. The products were pooled and annealed for 10 minutes at 90°C, 10 minutes at 4°C, and 2 hours at 40°C in a hybridization mixture containing NaCl 250 mM, Tris HCl 40 mM at pH 7.5 and EDTA 5 mM in RNase free water. The dsRNA was eluted using diethyl pyrocarbonate treated H₂O, and the integrity of the dsRNA was confirmed by running a 1% agarose gel in TBE 1X (90 mM Tris-borate/2 mM EDTA pH 8.0). It was then purified by column chromatography. See Tabs 6-7 attached hereto. See also, for example, the pages at Tab

8 showing an agarose gel showing the affect of dsRNA on c-Kit receptor signaling in HL-60 cells.

8. Cells were incubated under the same conditions, at 37°C., in 5% CO₂ for 1-4 days. See Tabs 8-10 attached hereto. At the end of the incubation period, FACS analyses were performed as described in the specification. Although no effect on KitR expression was observed until day 3, then inhibition was seen as disclosed in the specification, although only in the cells exposed to the KdsRNA. HL-60 cells behaved differently as shown at Tab 9, and as disclosed in the specification from CHP 100 cells, in both cases the KitR expression was unaffected by comparable doses of GdsRNA.

9. The foregoing experiment (actually a series of experiments) demonstrated for the first time that it is possible to disrupt gene expression at the mRNA level in a mammalian target gene in a *human* cell by providing to that human cell, interfering dsRNA (dsRNA) guide sequences that are homologous to a portion of the target gene, such that RNA interference (RNAi) of the target gene is induced. For example as shown, KitR expression was significantly reduced in human cancer cells treated with c-Kit dsRNA, but no reduction in KitR expression was seen upon treatment of the cells with GdsRNA. If the inhibition of KitR expression upon administration of c-Kit dsRNA had been due to a general inhibition of translation via the PKR response, then a similar reduction in KitR expression would have been induced by the GFP dsRNA. However, since KitR expression was reduced only upon administration of the target specific c-Kit dsRNA, the inhibition of KitR expression was necessarily due to gene-specific gene silencing (disrupted expression) as a result of the introduced dsRNA. Thus, the present specification, in describing and enabling the claimed invention, demonstrates the feasibility of inducing gene-specific RNAi in human cells with dsRNA, without apparent interference from the PKR response or other defenses.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patents issued thereon.

Respectfully submitted,

A handwritten signature in black ink, appearing to read 'Alan M. Gewirtz', written over a horizontal line.

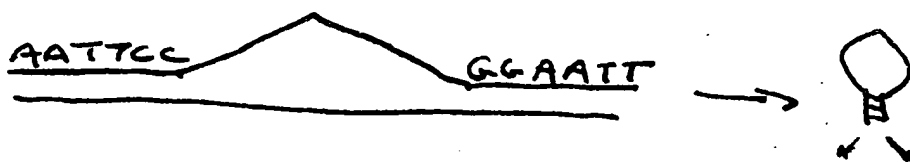
Date: April 28, 2006

Alan M. Gewirtz

1) C-KIT mRNA (cDNA) - FULL LENGTH CLONED INTO pCDNA3. → \Rightarrow RNA TRANSCRIPTS

2) TARGET RNA TRANSCRIPT & MOLECULAR BEACONS

3) SEQUENCE SELECTION FOR BEACONS?
COMPUTER PROGRAM DESIGNED TO
SEARCH FOR PALINDROMES SEPARATED
BY $\sim 15-20$ NTS



4) MIX RNA TRANSCRIPT + VARIOUS BEACONS →
PICK BEACONS WHICH OPEN WELL \Rightarrow FLUORESCENCE

5) TAKE BEACONS WHICH APPEAR OPTIMAL IN
VITRO + TEST IN VIVO (CELL LINES) —
✓ HYBRIDIZATION → MICROSCOPY
✓ BIOLOGY → ? INHIBIT CELL GROWTH

SHOAMIN

LESBETH

New Hybridization Protocol

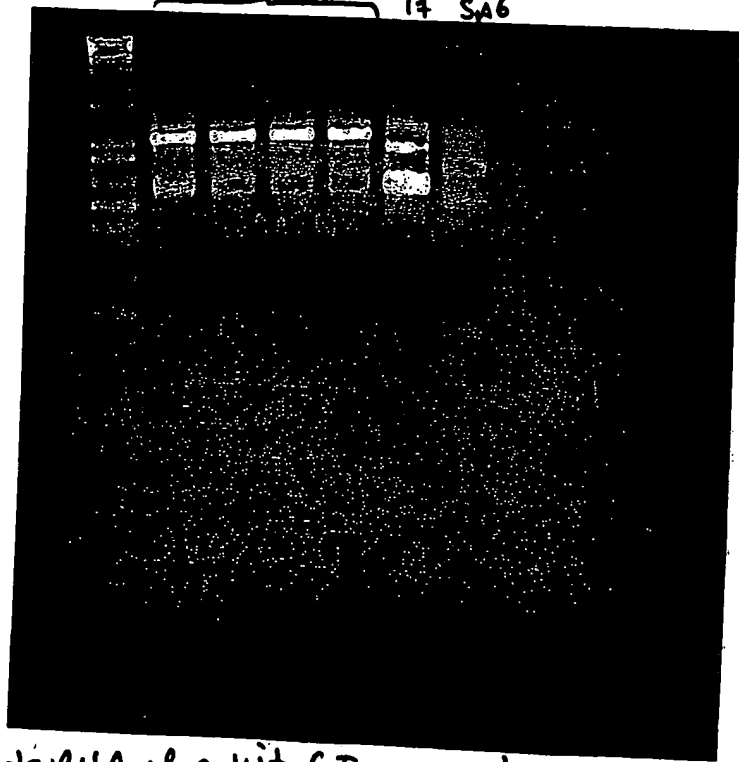
- 1- Purify RNA T7 and SAG with RNeasy kit
- 2- In Vitro Hybridization

SAG RNA 10 μ l
 T7 RNA 10 μ l
 1M NaCl 10 μ l
 200mM Tris 10 μ l (pH 7.5)
 25mM EDTA 10 μ l

In Vitro
 Hybridization
 buffer

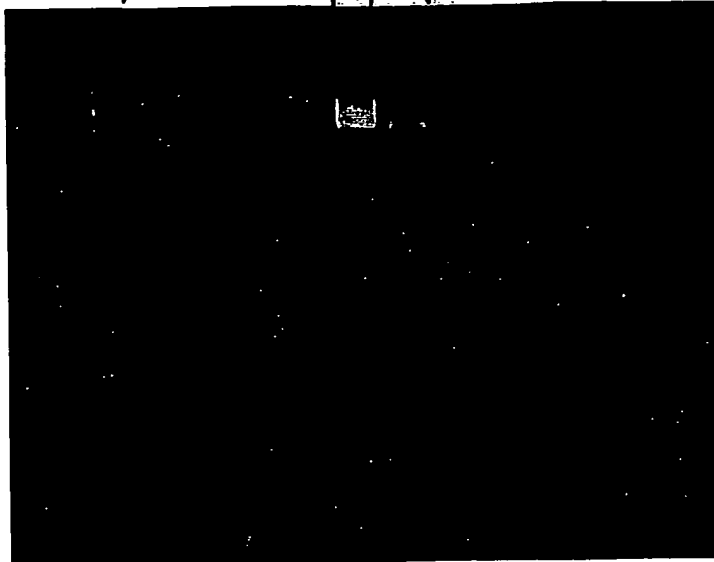
- 90°C 10 min
- On Ice Incubation 10 min.
- 40°C overnight Incubation

IVH(dsRNA) T7 SAG



dsRNA of c-kit (T7+SAG)

dsRNA T7 Sp6



dsRNA from c-kit

Confirmation of dsRNA with RNase digestion

1) RNase digestion

- 500 μ L 10X RNase digestion buffer
- 10 μ L RNase cocktail
- 440 μ L H₂O

500 μ L total volume

2) Samples

T7 20 μ L + 20 μ L dd H₂O = 50 μ L

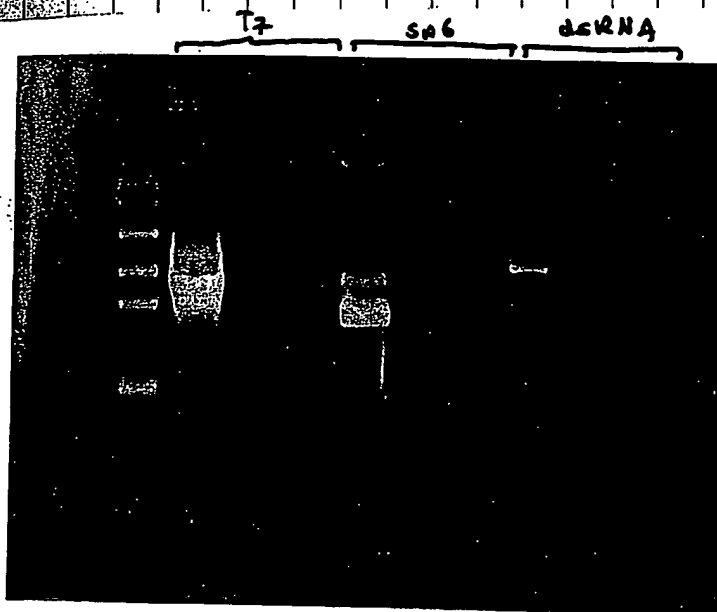
Sp6 20 μ L + 30 μ L dd H₂O = 4

dsRNA 20 μ L + 30 μ L dd H₂O = 4

3) 5 min 37°C Incubation

4) Anticollin by using (RNase kit)

5) Run on the 1% Agarose Gel



Before/After RNase treatment

RNase cocktail =

- RNase A 8.5 U/μL
- RNase T1 20 U/μL

Hybridization of SAG + T2 RNAs

a. Antipickup with RNase

b. SAG 10 μL

T2 10 μL

1M NaCl 10 μL

200 mM Tris 10 μL (pH 7.5)

25 mM EDTA 10 μL

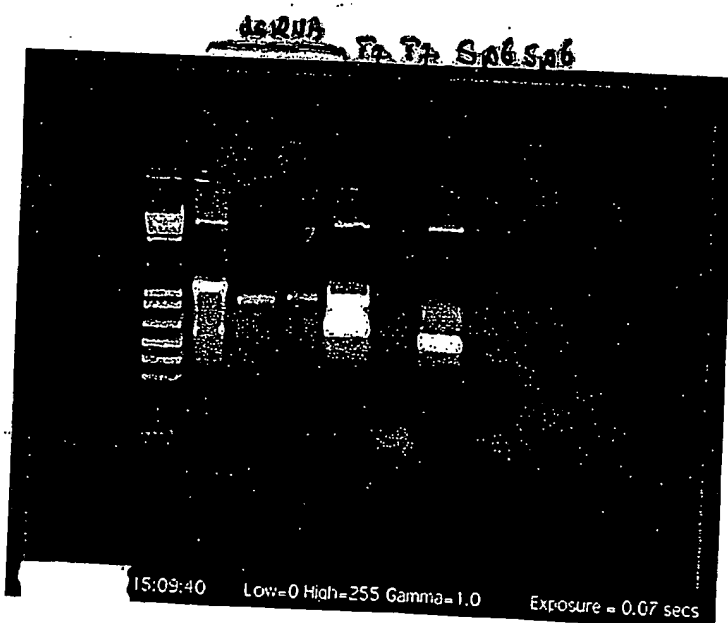
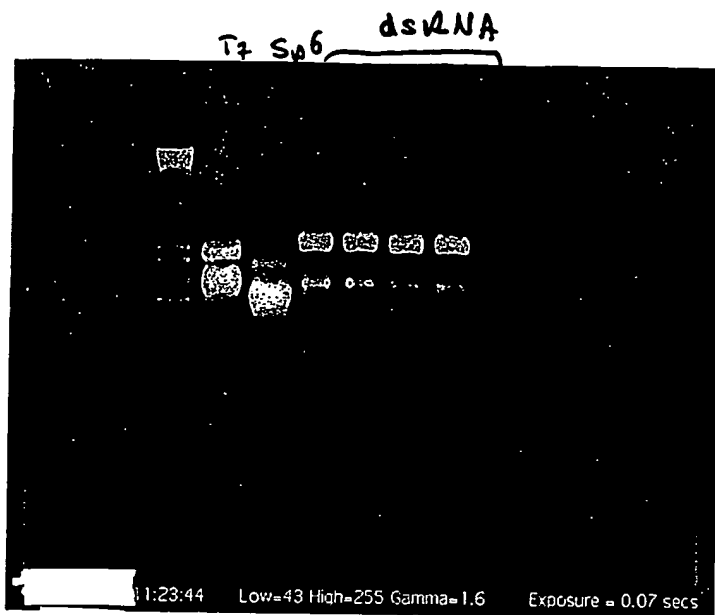
} in 4 tubes

c. 10 minutes 90°C

10 minutes on ice

Overnight 40°C

7.1



Treatment with RNase Cocktail

1-Check of the newly synthesized dsRNAs through
Agarose Gel & Spectrophotometry

2-New In Vitro Transcription reaction for Stock.

I/	T7	Sa6
	Abso mid 10 μ L	10 μ L
	dd H ₂ O 70 μ L	70 μ L
	Buffer 10 μ L	10 μ L (Napper NEB 2)
	Eco RI 10 μ L	10 μ L Hind III
	100 μ L	100 μ L

3hr in 37°C

I/ Removal of RNase

III/ RNA Purification (For ~~the~~ ^{the} reaction dd H₂O was used)

IV/ In Vitro Transcription

T7	Sa6
ANA. Reagents 40 μ L	50 μ L
Olipo's 30 μ L	20 μ L
Buffer 10X 20 μ L	20 μ L
Enzymes 10 μ L	10 μ L
100 μ L	100 μ L

3hr in 37°C

V/ Purification

CELL LINES WITH C-KIT EXPRESSION

1. Cell lines with high c-kit expression

CHU 100	(rhabdomyosarcoma)	c-kit
• WM 3P	(Melanoma)	% 94
SKCL-2	(Melanoma)	% 98
EW 9019	(Ovarian Ca)	% 79

2. Splitting of the cells

- Add 1ml Trypsin incubate 10min in 37°C
- Centrifuge 10min / 1000 rpm / +4°C
- Aspirate the supernatant.
- Add 2ml of the medium and mix through mixing
- Divide in different petry dishes (equal volume)
- Add ~~with~~ the medium to 10ml final volume

1- In Vitro Hybridization Experiments

- ① I select the tubes with incomplete hybridization
- ② 10min 90°C
- ③ 10min on Ice
- ④ overnight 40°C

2- Check with the %1 Appose Gel C



Molecular weight of RNA,

~~1000~~ 330 p/l

~~1000~~ 330 p/l

1 base = 330 p/l

1 base = 660 p/l

1 base = 0.66 p/ml

= 660 μ p/ml

1000 bases = 660.000 μ p/ml \Rightarrow 1M

" = 660 μ p/ml \Rightarrow 1 μ M.

" 660 μ p/ml \Rightarrow 1 μ M

#) First Incubation Experiment with ds RNA

Init. Cont. 24st 48st

①

94%

1ml

1ml

24st

1ml

48st

1ml

72st

CHW-100 (1 μ M of dsRNA)
(\approx 600 μ p)

1 μ M of dsRNA = 10 μ l in 1ml

94%

1ml

1ml

1ml

WM 3P

94%

1ml

1ml

1ml

SKCL-2

1ml

1ml

1ml

1ml

CW 9019

1 μ M dsRNA
(600 μ p)

FACS analysis at 24 and 48 hr.

Experiment # 1 (24 hr)

Experiment # 2 (48 hr)

New In Vitro Hybridization Experiment

(I)

Amu KIT2/EcoRV
(T2)

Amu KIT2/Hind III
(SAB)

Alcosmid	10 μ l
dd H ₂ O	70 μ l
10X Supper	10 μ l
Enzyme	10 μ l (EcoRV)
<hr/>	
	100 μ l

10 μ l	
70 μ l	
10 μ l	
10 μ l (Hind III)	
<hr/>	
100 μ l	

* Inc in 37°C

(II) Removal of RNAse (1/1)

- 50 μ l RNasease Product
- 50 μ l RNasease Rem Solution

(III) PCR Amplification

(IV) In Vitro Transcription

T2 directed

SAB directed

ONA	40 μ l
oligo's	30 μ l
Supper 10X	20 μ l
Enzyme mix	10 μ l
<hr/>	
	100 μ l

50 μ l	
20 μ l	
20 μ l	
10 μ l	
<hr/>	
100 μ l	

* Inc in 37°C

(V) In Vitro Hybridization

T2 RNA	50 μ l
SAB "	50 μ l
1X NOC	50 μ l

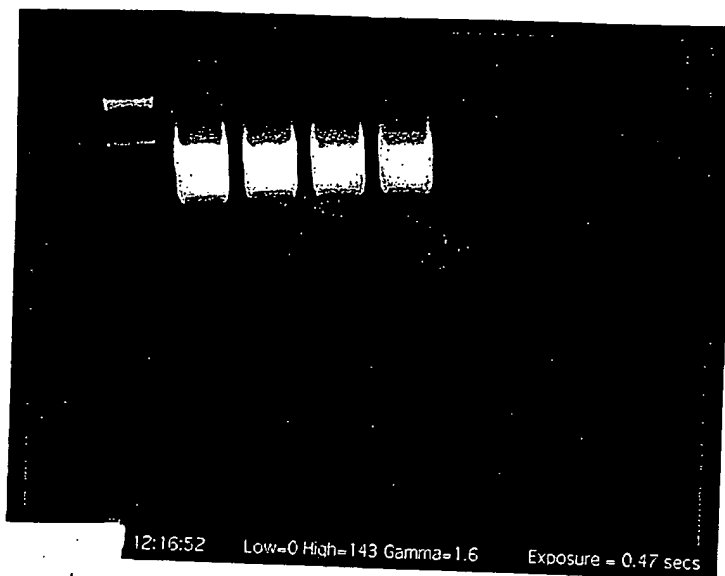
20 μ l	20 μ l	50 μ l
25 μ l	25 μ l	10 μ l
15		

15 min 90°C

15 min on ice

Overnight 40°C

(vi) Purification of dsDNA



dsDNA for c-kit

dsDNA

EMERSON LAB

Time: 13:43

DNA/Oligo Quant

ReadSamples RawData Method SaveClear Print Quit

Results file: A:\WORK_RES

Method name: A:\DS_DNA

Assay type: ds DNA

Sampling device: None

Read average time: 0.50 sec

Background corr: [Yes] 320.0nm

Pathlength: 1.0000 cm

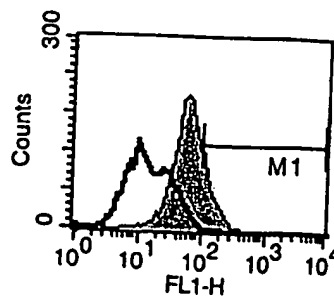
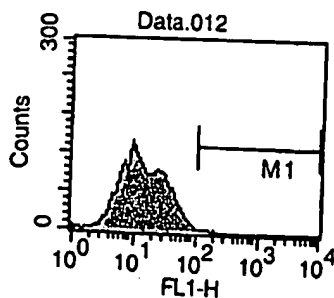
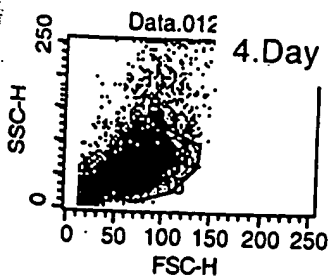
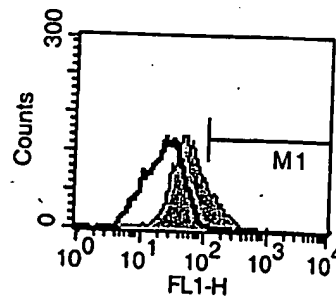
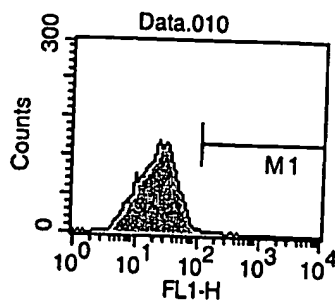
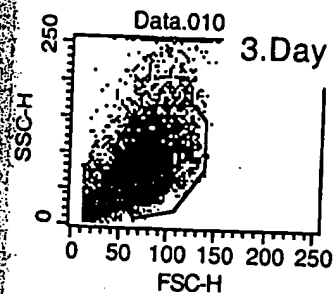
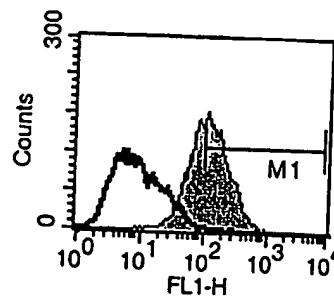
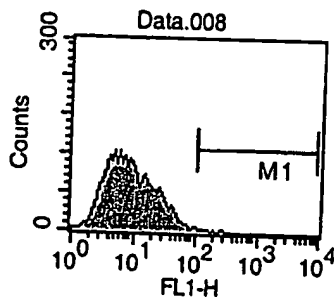
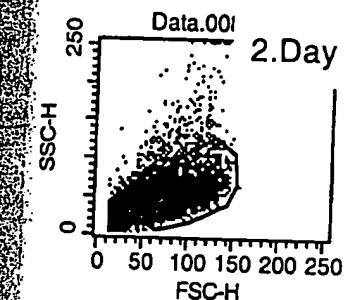
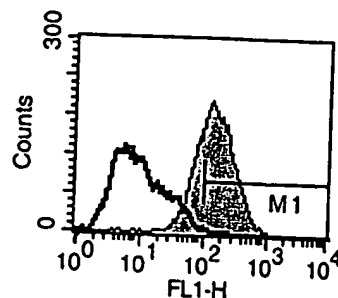
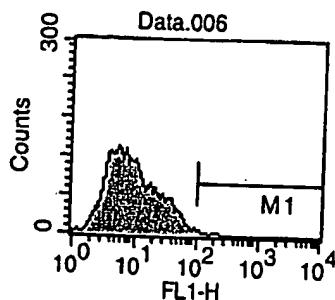
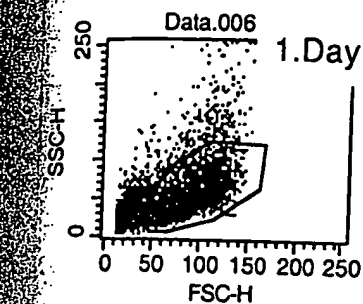
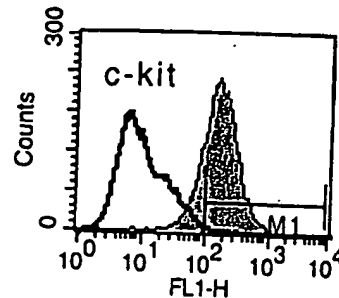
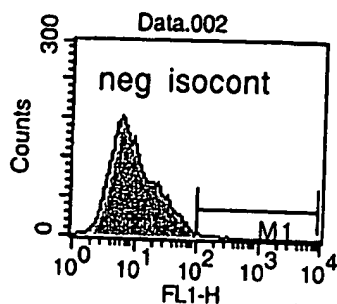
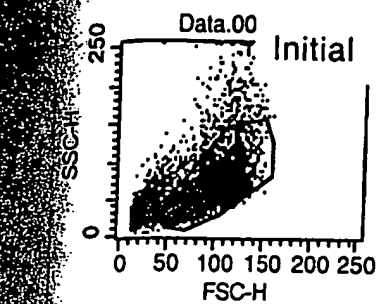
Conc. factor: 50.000 at 260.0nm ↓ ↑

Sample ID	Net Abs 260.0nm	Net Abs 280.0nm	260.0/280.0	Dil. Fact.	Conc. ug/mL
1	2.2019	1.1041	1.9942	50.000	5504.6992
2	2.1363	1.0397	2.0548	50.000	5340.8506
3				50.000	

} 2 sample total vol.

US Appl. No.: 09/993,183
000011

CHP-100 Rbdomvsarcoma cell line



Histogram Statistics

File: Data.003

Log Data Units: Linear Values

Sample ID:

Patient ID:

Tube:

Panel:

Gate: G1

Gated Events: 8108

Total Events: 10000

X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	8108	100.00	81.08	188.93	159.48	117.84	62.37	159.63	232	159
M1	100, 8660	6493	80.08	64.93	217.94	196.16	114.16	52.38	184.34	232	159

Histogram Statistics

File: Data.007

Log Data Units: Linear Values

Sample ID:

Patient ID:

Tube:

Panel:

Gate: G2

Gated Events: 8093

Total Events: 10000

X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	8093	100.00	80.93	169.40	140.31	110.77	65.39	138.24	204	128
M1	100, 9647	5753	71.09	57.53	209.57	188.68	107.46	51.27	177.83	204	128

Histogram Statistics

File: Data.009

Log Data Units: Linear Values

Sample ID:

Patient ID:

Tube:

Panel:

Gate: G3

Gated Events: 7345

Total Events: 10000

X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	7345	100.00	73.45	137.97	112.61	94.27	68.32	111.40	181	111
M1	100, 8660	4218	57.43	42.18	191.55	174.85	91.91	47.98	165.48	181	111

Histogram Statistics

File: Data.011

Log Data Units: Linear Values

Sample ID:

Patient ID:

Tube:

Panel:

Gate: G4

Gated Events: 5102

Total Events: 10000

X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	5102	100.00	51.02	76.80	59.85	146.80	191.14	56.23	144	43
M1	111, 9647	920	18.03	9.20	185.82	165.99	320.68	172.58	153.99	56	138

Histogram Statistics

File: Data.013

Log Data Units: Linear Values

Sample ID:

Patient ID:

Tube:

Panel:

Gate: G5

Gated Events: 6611

Total Events: 10000

X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	6611	100.00	66.11	70.83	60.95	42.17	59.53	60.43	206	54
M1	100, 9647	1241	18.77	12.41	140.08	134.79	43.27	30.89	124.09	123	100

① dsRNA for c-kit and GFR

② Linearization

shuKIT2

T7, EcoRV

SAB: HindIII

GFR

T7, EcoRV

SAB: HindIII

③ Treatment with RNase Rem Mix

④ RNA Purification Kit

⑤ In vitro Transcription by using Promoter ~~in~~ Thermo
cycler (Gokhan / RNA1 (2hr incubation)
RNA2 (1hr 9)

⑥ In vitro Hybridization

SAB: 10 μ L

T7: 10 μ L

Nase 150 mM 20 μ L

50 μ L

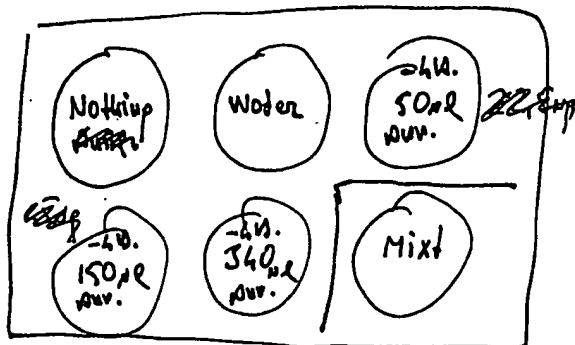
⑦ Purification of RNA

Experiment 6

Different Concent / vs Amplified ~~recovery~~

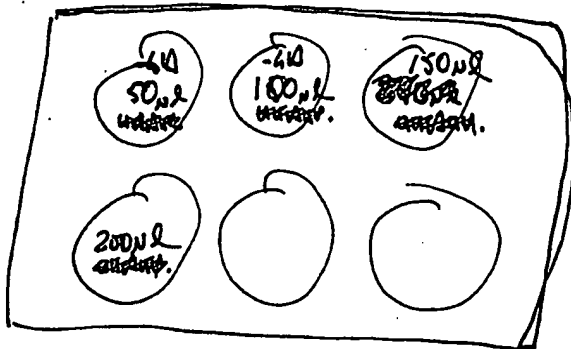
(6 h incubation)

CHW 100 Cells



Box 1

17/5/00



Box 2

EMERSON LAB

Time: 13:15

DNA/Oligo Quant

ReadSamples

RawData

Method

SaveClear

Print

Qui

Results file: A:\WORK_RES

Method name: A:\DS_DNA

Assay type: ds DNA

Sampling device: None

Read average time: 0.50 sec

Background corr: [Yes] 320.0nm

Pathlength: 1.0000 cm

Conc. factor: 50.000 at 260.0nm + +

Sample ID	Net Abs 260.0nm	Net Abs 280.0nm	260.0/280.0	Dil. Fact.	Conc. ug/mL
1	1.5477	0.8350	1.8535	50.000	3869.3638
2	1.5660	0.8373	1.8703	50.000	3915.0818
3				50.000	

1. Creation of a new ds RNA from AhuKIT2:

a) Linearization

T7 (EcoRV)		Sag (HindIII)	
Absmid	10 μ l		10 μ l
dd H ₂ O	70 μ l		70 μ l
Buffer 10X	10 μ l	NEB2	10 μ l
EcoRV	10 μ l	HindIII	10 μ l
100 μ l		100 μ l	

b) Inactivation of RNase

50 μ l Rnased Absmid
 50 μ l Mixture (with RNasease K)
 37°C 15 min

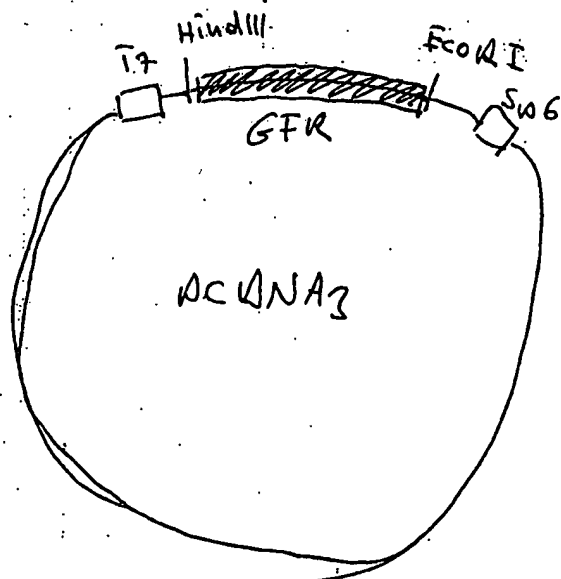
c) In vitro Transcription

d) Hybridization In vitro

e) Amplification of the ds RNA

* As a control for c-kit dsRNA, in lab meeting has been discussed and decided to use ~~GFP~~ GFP dsRNA

* Group provided ~~dsGFP~~ dsDNA3 with GFP insert
linearization of dsDNA3 with GFP insert



linearization
T7 → EcoRV
Sma6 → HindIII

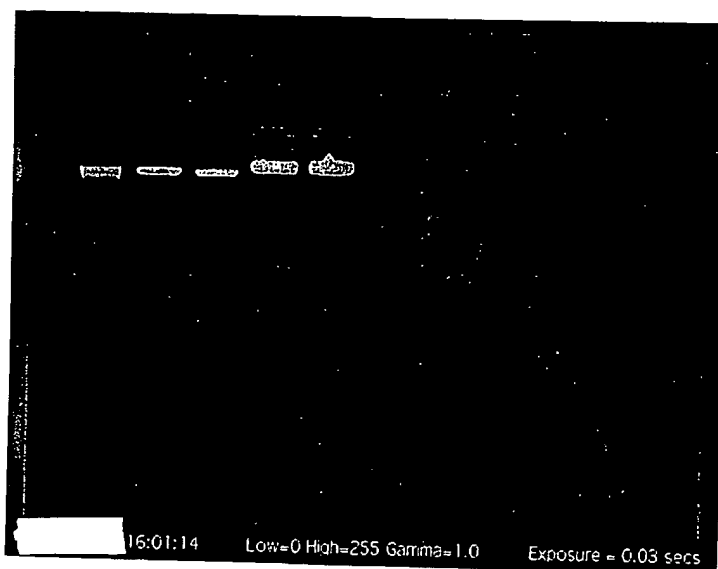
T₂ Circulation

Alcosmid	1ul
dd H ₂ O	7ul
10X TAP	1ul
EcoRV	1ul
	<hr/> 10ul

Sub Circulation

	1ul
	7ul
	1ul
HindIII	1ul
	<hr/> 10ul

Running on the 1% Agarose Gel



A 1: ACUNA3 + KIT / EcoRV L4: ACUNA3 + GFA / HindIII
 2: ACUNA3 + KIT / HindIII
 3: ACUNA3 + GFA / EcoRV

Double Strand RNA for GFP

1) Linearization

T7
Alosmid 10 μ l
dd H₂O 70 μ l
Buffer 10X 10 μ l
EcoRV 10 μ l

100

SAB
10 μ l
70 μ l
10 μ l
HindIII 10 μ l

100 μ l

3hr at 37°C

2) RNase Treatment

3) Purification of the template

4) In Vitro Transcription

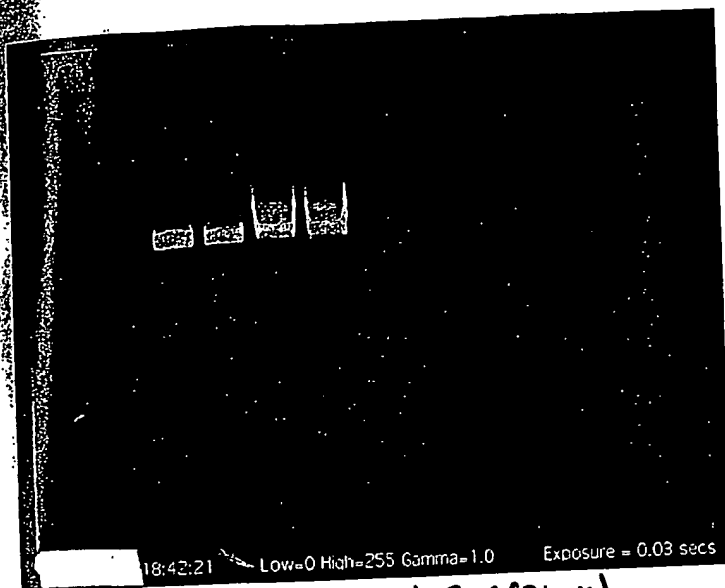
T7
Template 40 μ l
Oligo 30 μ l
Buffer 10X 20 μ l
Enzyme mix 10 μ l

SAB
50 μ l
20 μ l
20 μ l
10 μ l

3hr at 37°C

5) Gel Confirmation ~~Confirmation~~

6) Purification



GFW ssRNA T7 (2 lane) SA6 (2 lane)

g) In vitro Hybridization Experiment of GFW ssRNA's

10ul	T7 ssRNA
10ul	SA6 ssRNA
10ul	1M NaCl
10ul	25 mM EDTA
10ul	200 mM Tris HCl (pH 7.5)

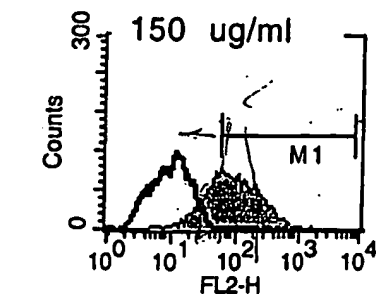
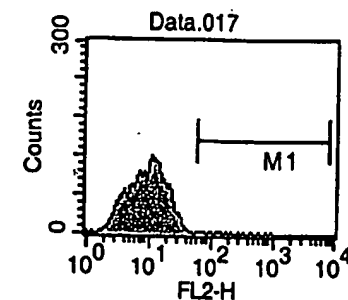
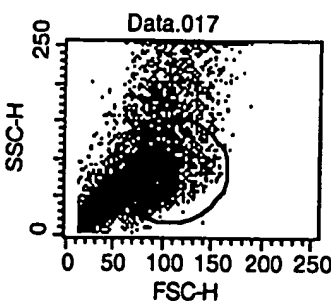
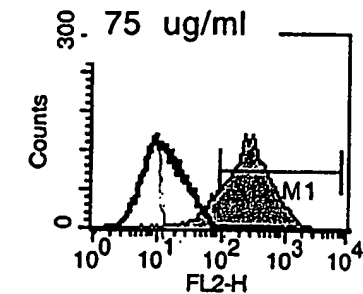
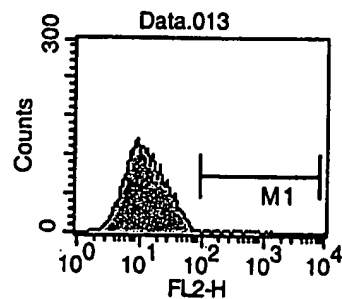
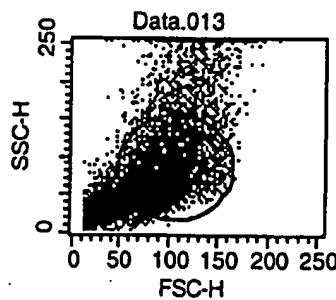
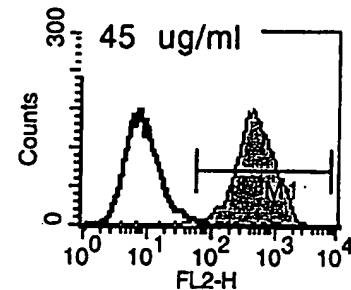
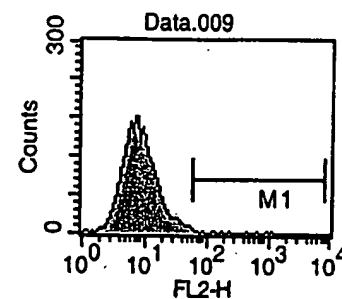
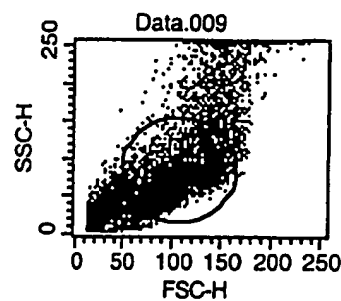
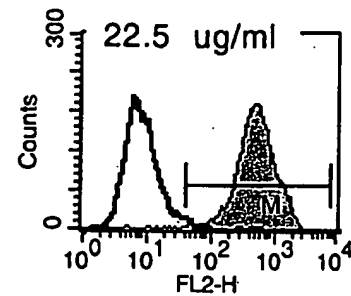
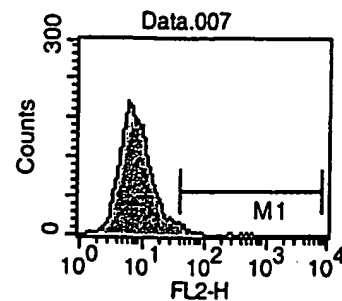
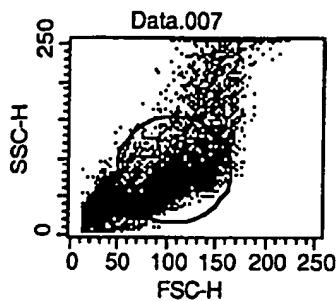
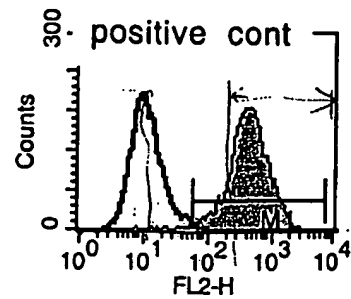
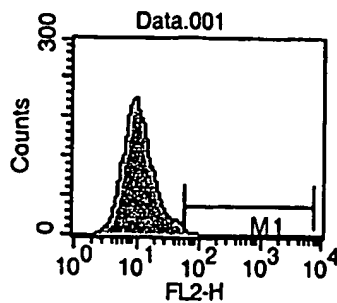
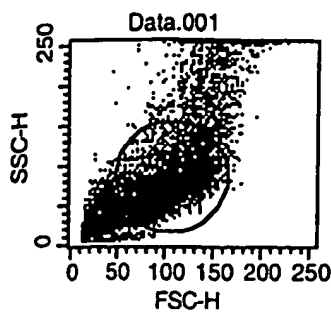
50ul.

80°C	90°C	10 minutes
	On Ice	10 minutes
	40°C	3hr

7) Aposome Gel Confirmation

8) Purification.

CHP 100 cell Line experiment#6 (different concentrations of dsRNA)



Histogram Statistics

File: Data.002

Sample ID:

Tube:

Gated Events: 7279

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	7279	100.00	72.79	483.30	395.71	312.72	64.71	406.79	184	378
M1	58, 7234	7230	99.33	72.30	486.29	401.90	311.67	64.09	406.79	184	378

Histogram Statistics

File: Data.008

Sample ID:

Tube:

Gated Events: 7279

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	7279	100.00	72.79	569.13	469.90	361.54	63.52	469.76	192	486
M1	41, 7774	7262	99.77	72.62	570.41	473.35	361.00	63.29	469.76	192	486

Histogram Statistics

File: Data.010

Sample ID:

Tube:

Gated Events: 6960

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	6960	100.00	69.60	559.17	458.14	359.82	64.35	453.16	179	421
M1	58, 7774	6928	99.54	69.28	561.57	463.53	358.92	63.91	453.16	179	421

Histogram Statistics

File: Data.014

Sample ID:

Tube:

Gated Events: 6348

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	6348	100.00	63.48	328.52	241.42	273.62	83.29	254.83	142	283
M1	93, 7774	5538	87.24	55.38	367.24	295.88	272.06	74.08	283.87	142	283

Histogram Statistics

File: Data.018

Sample ID:

Tube:

Gated Events: 4716

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

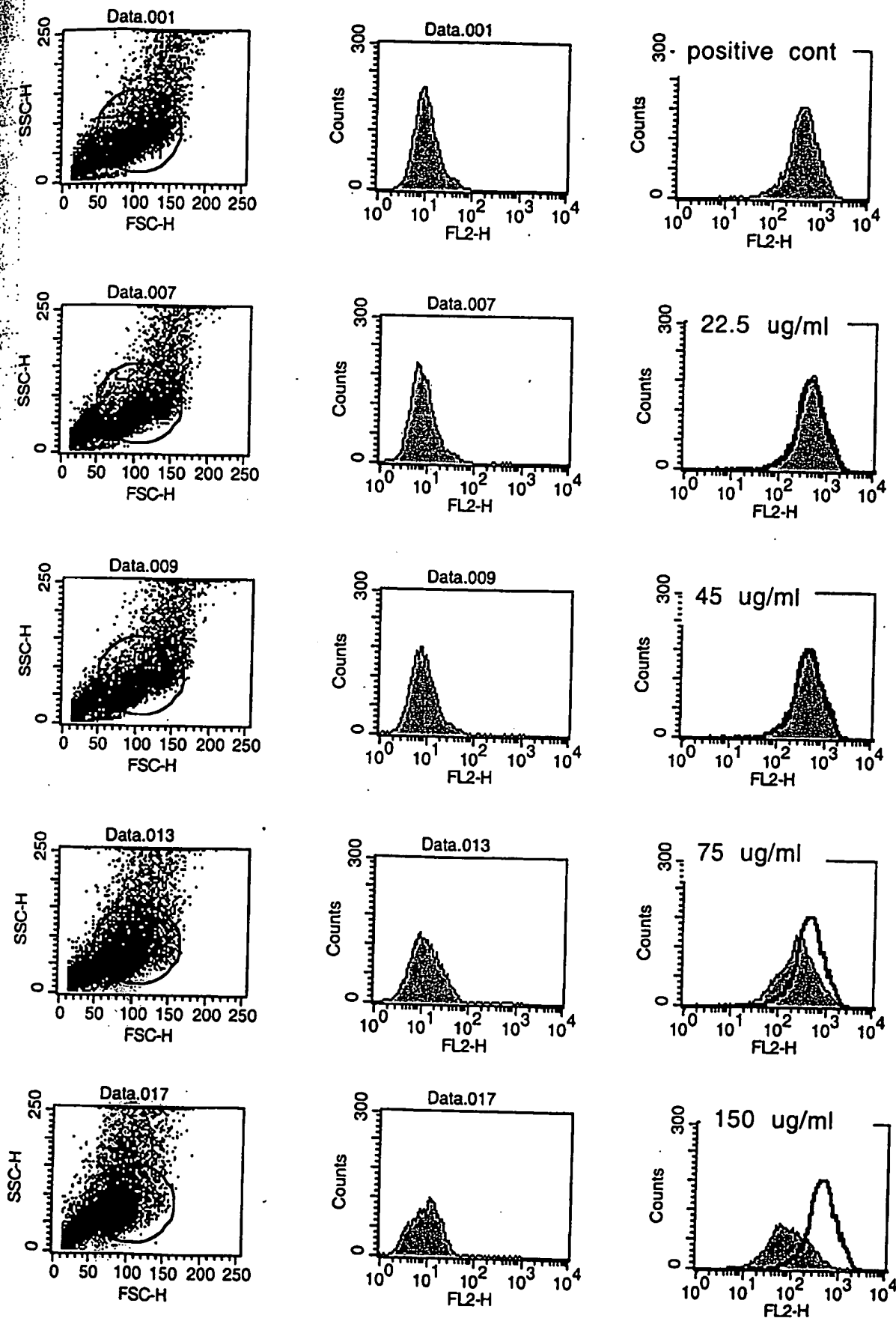
Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch.
All	1, 9647	4716	100.00	47.16	125.25	88.60	116.68	93.16	86.60	89	58
M1	58, 7774	3219	68.26	32.19	166.16	136.83	120.86	72.74	124.09	89	58

CHP 100 cell Line experiment#6 (different concentrations of dsRNA)



Experiment # 8 / Flow

1. Effect of anti-c-kit dsRNA to c-kit expression in HL60 cells.

1ml (100,000 cells)

1ml " 150 NP 75 NP

1ml " 150 NP 150 NP

1ml " 225 NP

1ml " 300 NP

* Treatment

* FACS.

3 Day

Experiment 8 : dsRNA for GFA / CHA 100

Control 50 75
150 250

1. In vitro transcription for c-kit

a. Plasmid linearization

<u>T7</u>		<u>Sp6</u>	
Plasmid	10 μ L	10 μ L	
ddH ₂ O	70 μ L	70 μ L	
NE Buffer 3	10 μ L	NE Buffer 2	10 μ L
EcoRV	10 μ L	Hind III	10 μ L
<hr/> 100 μ L		<hr/> 100 μ L	

3hr @ 37°C

b. Treatment for RNase

50 μ L Digestion Product / mix well
50 μ L Mixture

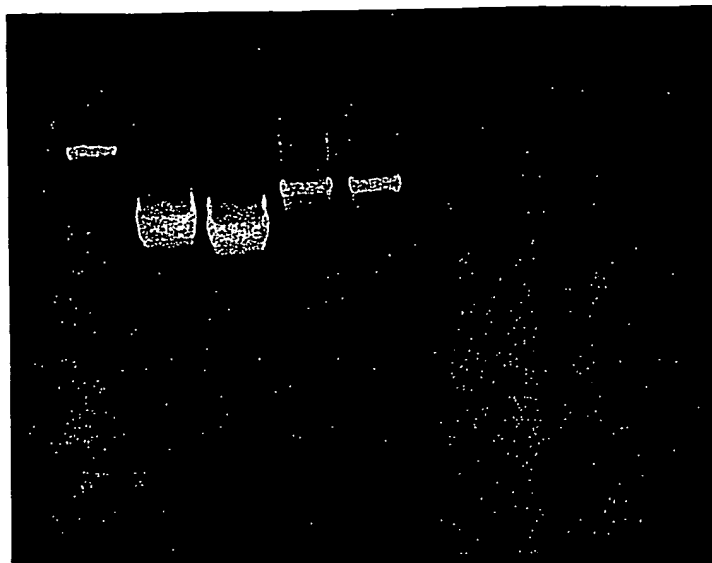
1 1/2 hr @ 37°C

c. RCL Purification Kit (Final Volume 60 μ L)

d. In vitro Transcription

	<u>T7</u>	<u>Sp6</u>
Template	60	50
NTAs	30	20
Buffer EX	20	20
Fugue mix	10	10
<hr/> 100 μ L		<hr/> 100 μ L

3hr @ 37°C



dsRNA of KIT
T7/Su6/dsRNA

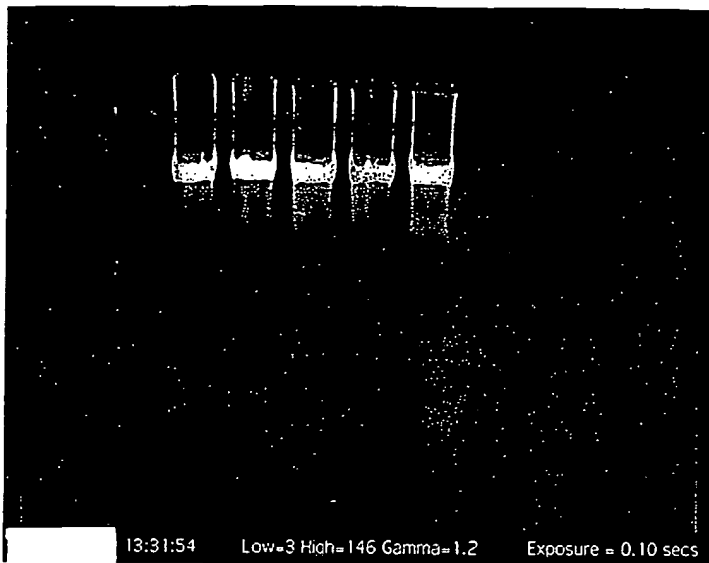


19:01:36 Low=10 High=164 Gamma=1.2 Exposure = 0.10 secs

In vitro hybridization

C1: Mixture 1

C2: " 2 (only 150 uM NaCl)



KIT ds RNA

7/27/00

FACS (GFW in CAW-100, KIT Hc 60)

1. In Vitro Transcription Reaction

2.



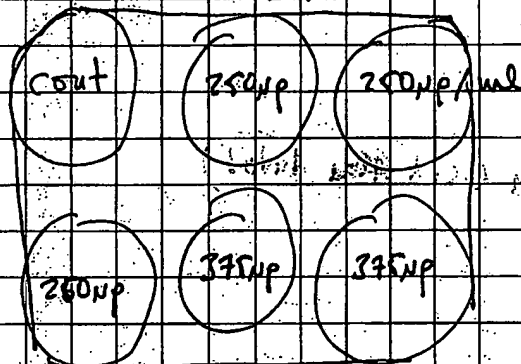
In Vitro Hybridization by using
Thermocycler.



~~SS RNA~~ ss RNA of c-kit (Ahv KIT2)
 2L = T₂ directed 2L = S₄₆ directed
 8/2/00

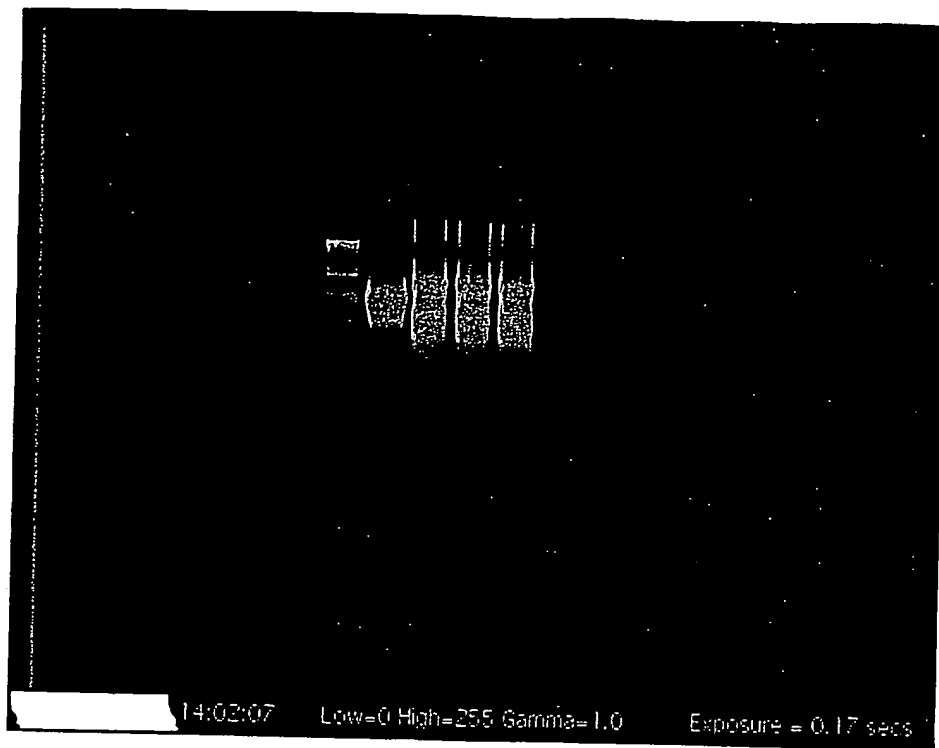
Experiment 10

Ahv KIT ds RNA



Incubation time 3 days

FACS Analysis

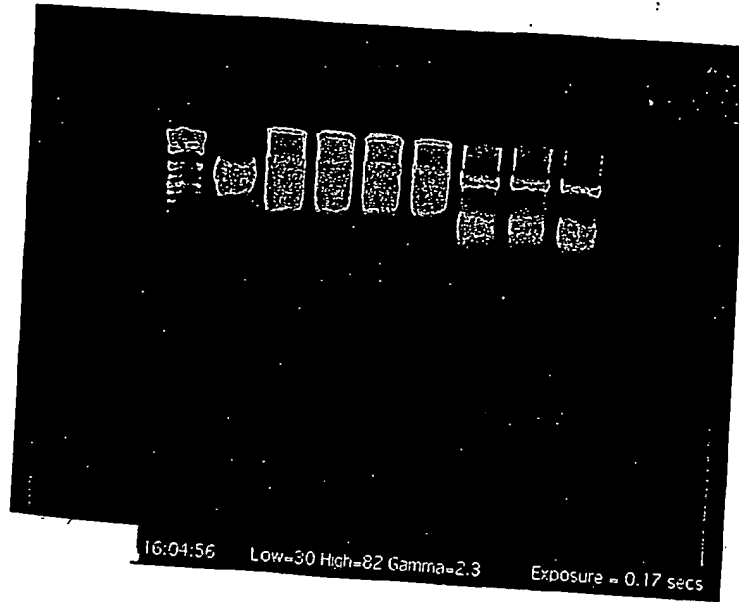


Incomplete Hybridization ?

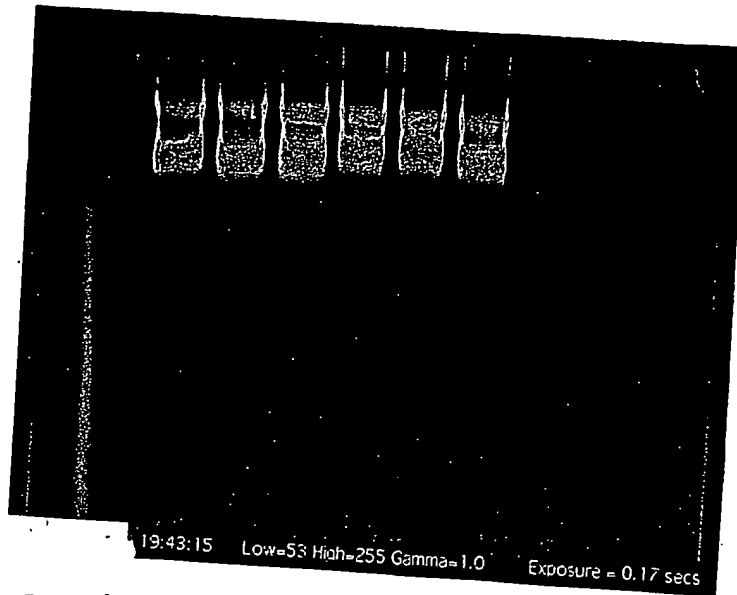
New Hybridization Protocol

- ① 10min at 90°C Thermocycler (cid ~~for~~ Auto!)
- ② " at 4°C (ov ice)

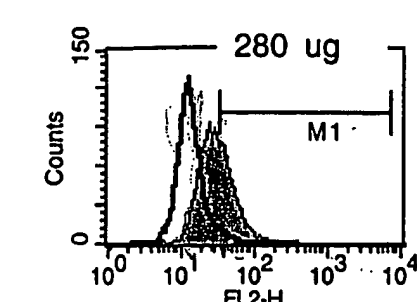
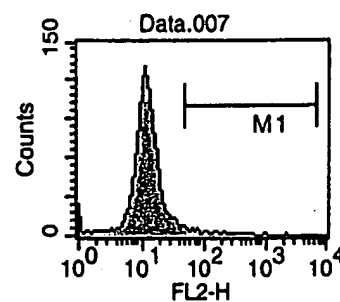
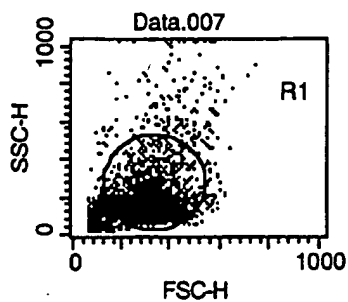
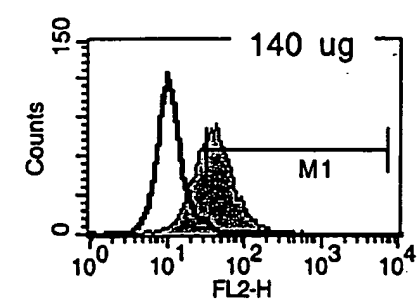
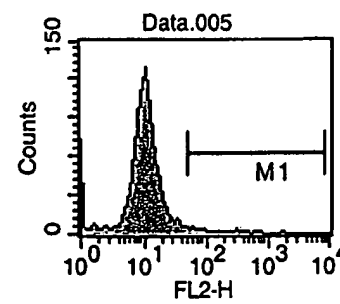
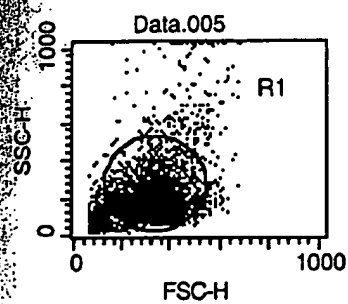
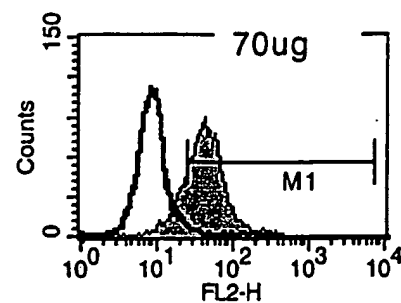
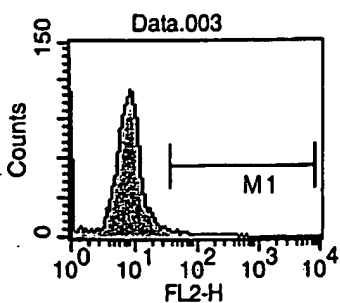
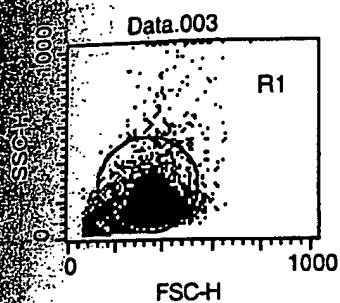
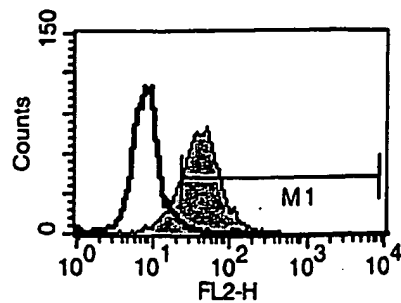
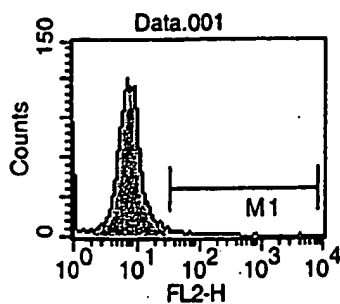
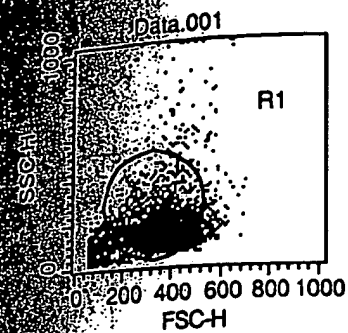
(00/1/2) 2nd 2nd notations
(00/1/2) 2nd 2nd



In Vitro Hybridization (New Method)



In Vitro Hybridization (New Method)



Histogram Statistics

File: Data.002

Sample ID:

Tube:

Gated Events: 8755

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	8755	100.00	87.55	44.09	38.24	61.48	38.54	46
M1	23, 8505	7379	84.28	73.79	49.03	44.40	54.51	42.55	46

Histogram Statistics

File: Data.004

Sample ID:

Tube:

Gated Events: 9206

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9206	100.00	92.06	45.65	39.67	62.07	39.95	41
M1	26, 7499	7433	80.74	74.33	51.86	47.24	54.18	44.91	41

Histogram Statistics

File: Data.006

Sample ID:

Tube:

Gated Events: 9249

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9249	100.00	92.49	44.26	38.28	62.80	38.20	40
M1	32, 7365	5846	63.21	58.46	56.45	52.10	50.11	48.26	40

Histogram Statistics

File: Data.008

Sample ID:

Tube:

Gated Events: 9379

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

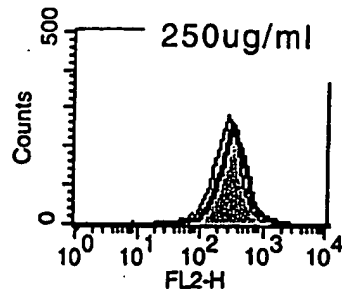
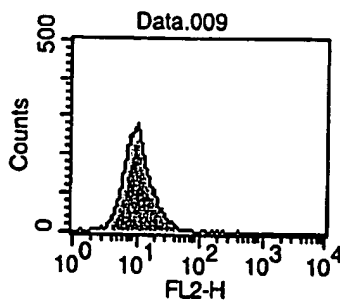
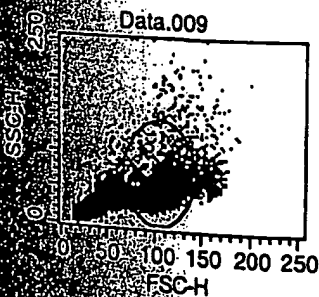
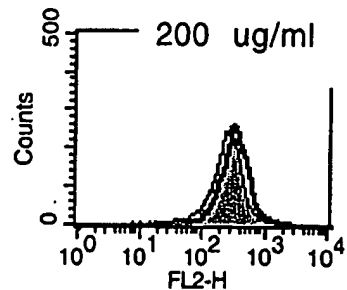
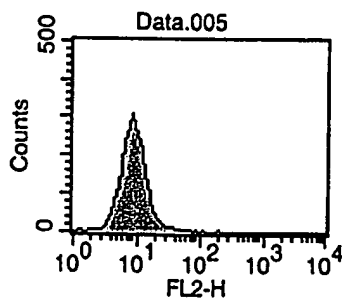
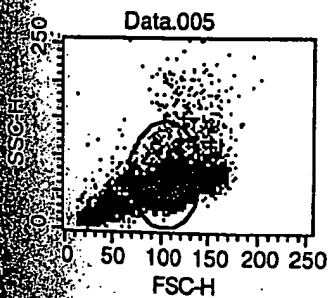
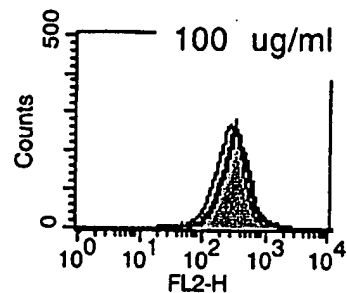
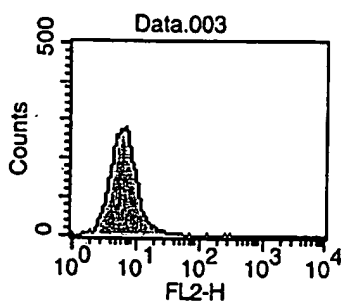
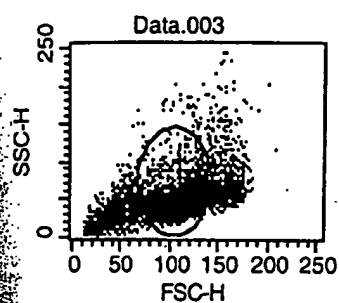
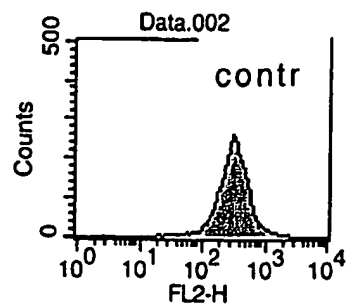
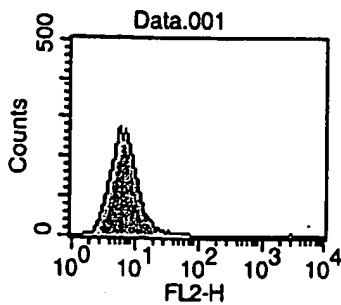
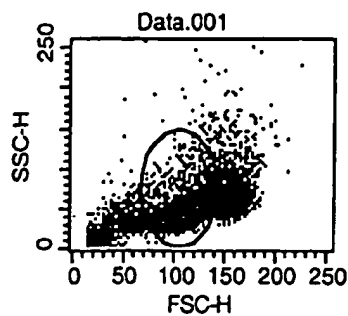
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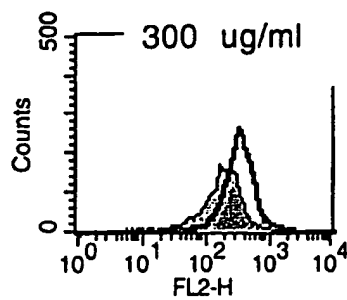
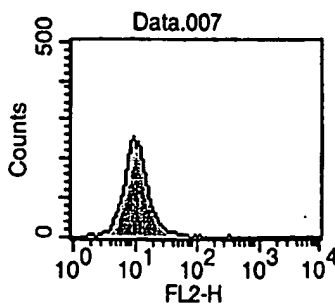
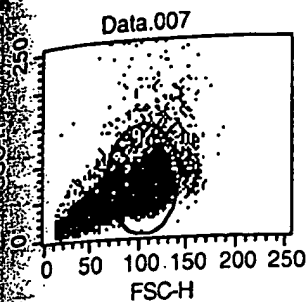
Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9379	100.00	93.79	31.09	27.69	58.20	26.66	22
M1	32, 7041	3342	35.63	33.42	47.81	45.13	43.51	41.79	34

EFFECT OF dsRNA of GFP to c-kit expression on CHP 100 Cell Line
DAY 3 (Experiment 4)





Histogram Statistics

File: Data.002

Sample ID:

Tube:

Gated Events: 7042

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9647	7042	100.00	70.42	339.23	295.61	57.86	305.05	283

Histogram Statistics

File: Data.004

Sample ID:

Tube:

Gated Events: 7599

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9647	7599	100.00	75.99	273.39	244.26	48.98	254.83	327

Histogram Statistics

File: Data.006

Sample ID:

Tube:

Gated Events: 6794

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9647	6794	100.00	67.94	260.94	229.14	50.57	245.82	273

Histogram Statistics

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

File: Data.010

Sample ID:

Tube:

Gated Events: 7563

X Parameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9647	7563	100.00	75.63	272.39	242.09	49.33	254.83	273

Histogram Statistics

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G1

Total Events: 10000

File: Data.008

Sample ID:

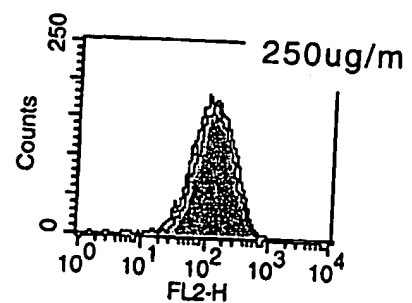
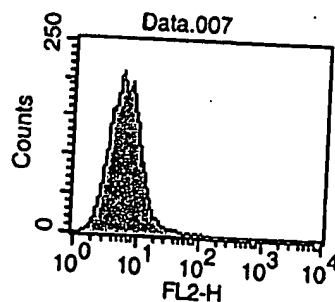
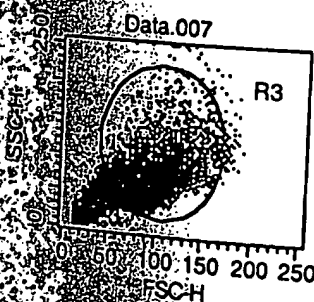
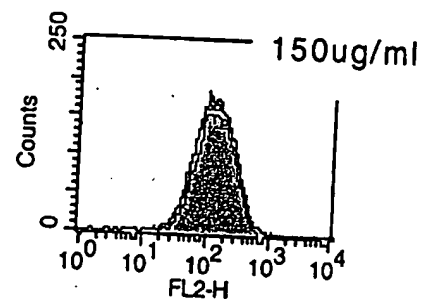
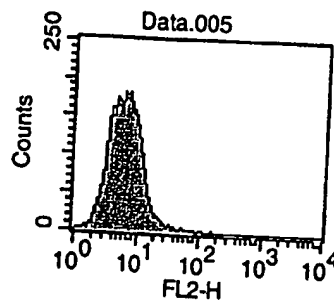
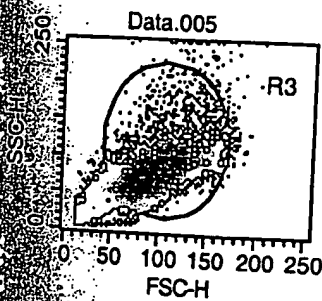
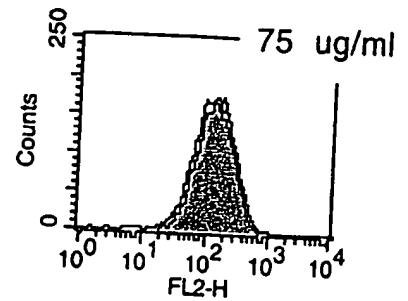
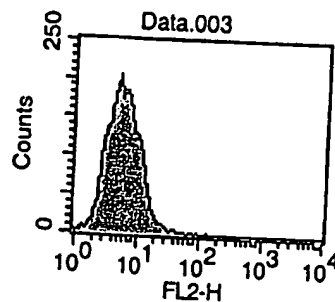
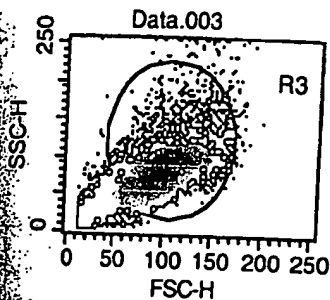
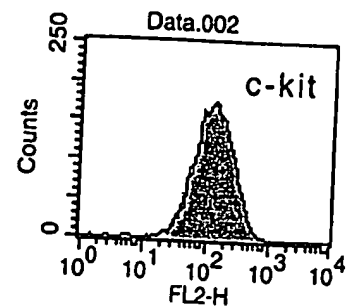
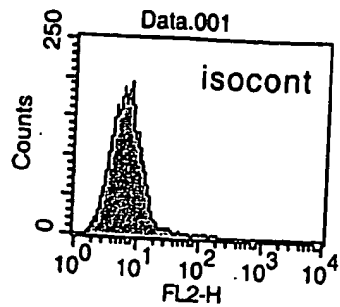
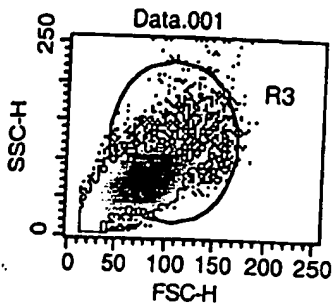
Tube:

Gated Events: 5279

X Parameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9647	5279	100.00	52.79	195.58	166.36	54.93	177.83	159

CHP 100 Cell Line dsRNA for GFP 3.Day Incubation (Experiment 8)



Histogram Statistics

File: Data.002

Sample ID:

Tube:

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G3

Total Events: 10000

Gated Events: 7199

XParameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9647	7199	100.00	71.99	144.92	119.41	64.54	119.71	124

Histogram Statistics

File: Data.004

Sample ID:

Tube:

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G3

Total Events: 10000

Gated Events: 7610

XParameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9647	7610	100.00	76.10	150.33	123.71	65.56	124.09	171

Histogram Statistics

File: Data.006

Sample ID:

Tube:

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G3

Total Events: 10000

Gated Events: 7491

XParameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9647	7491	100.00	74.91	154.69	125.48	67.86	124.09	96

Histogram Statistics

File: Data.008

Sample ID:

Tube:

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G3

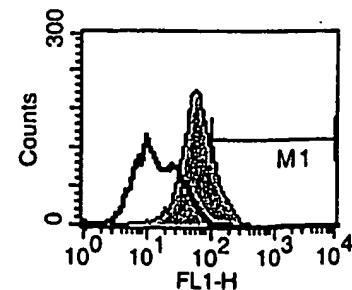
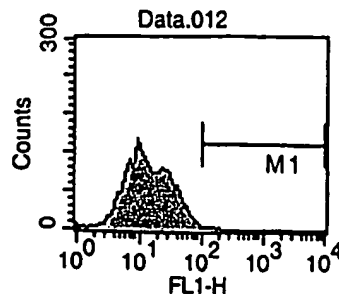
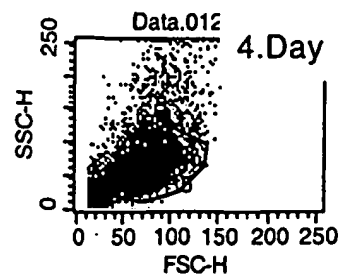
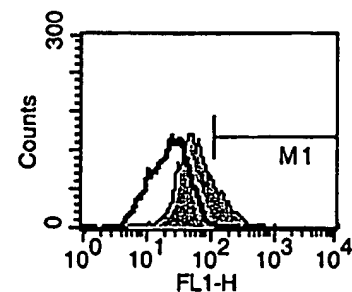
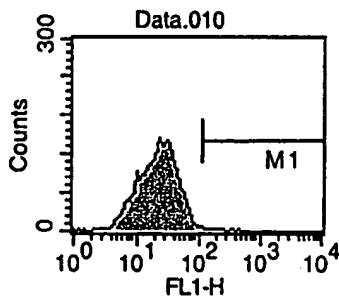
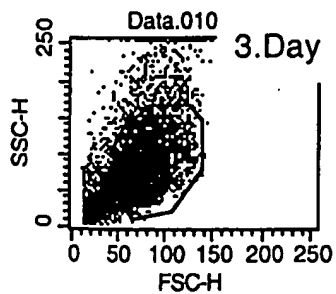
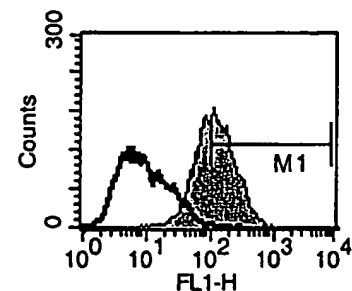
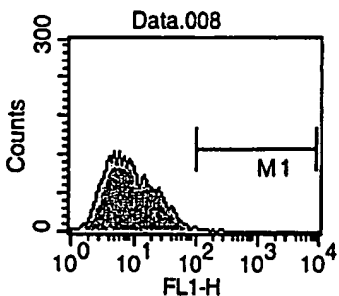
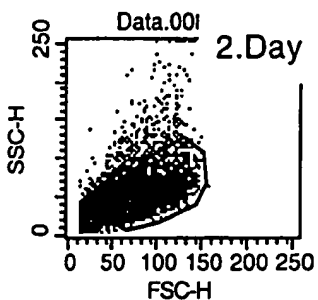
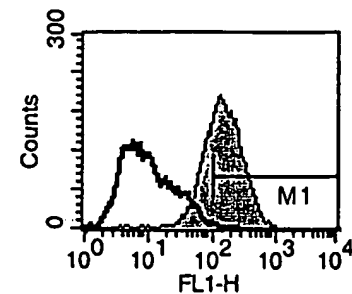
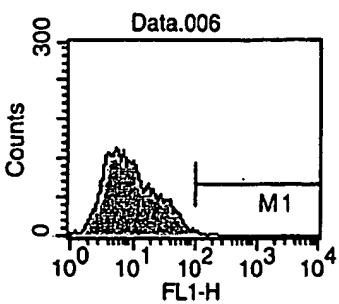
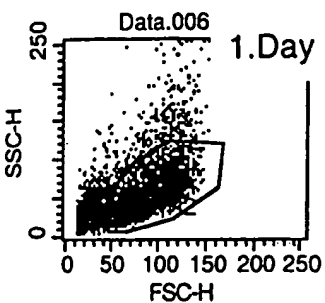
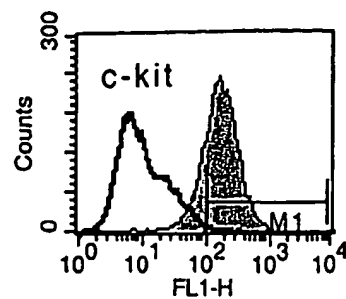
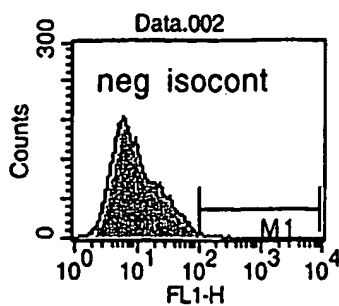
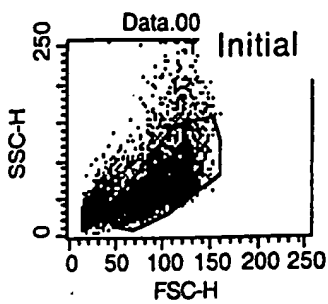
Total Events: 10000

Gated Events: 7355

XParameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9647	7355	100.00	73.55	139.23	113.40	67.16	111.40	100

CHP-100 Rbdomvsarcoma cell line



Histogram Statistics

File: Data.003

Log Data Units: Linear Values

Sample ID:

Patient ID:

Tube:

Panel:

Gate: G1

Gated Events: 8108

Total Events: 10000

X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	8108	100.00	81.08	188.93	159.48	117.84	62.37	159.63	232	159
M1	100, 8660	6493	80.08	64.93	217.94	196.16	114.16	52.38	184.34	232	159

Histogram Statistics

File: Data.007

Log Data Units: Linear Values

Sample ID:

Patient ID:

Tube:

Panel:

Gate: G2

Gated Events: 8093

Total Events: 10000

X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	8093	100.00	80.93	169.40	140.31	110.77	65.39	138.24	204	128
M1	100, 9647	5753	71.09	57.53	209.57	188.68	107.46	51.27	177.83	204	128

Histogram Statistics

File: Data.009

Log Data Units: Linear Values

Sample ID:

Patient ID:

Tube:

Panel:

Gate: G3

Gated Events: 7345

Total Events: 10000

X Parameter: FL1-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	7345	100.00	73.45	137.97	112.61	94.27	68.32	111.40	181	111
M1	100, 8660	4218	57.43	42.18	191.55	174.85	91.91	47.98	165.48	181	111

Histogram Statistics

File: Data.011

Sample ID:

Tube:

Gated Events: 5102

X Parameter: FL1-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G4

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	5102	100.00	51.02	76.80	59.85	146.80	191.14	56.23	144	43
M1	111, 9647	920	18.03	9.20	185.82	165.99	320.68	172.58	153.99	56	138

Histogram Statistics

File: Data.013

Sample ID:

Tube:

Gated Events: 6611

X Parameter: FL1-H (Log)

Log Data Units: Linear Values

Patient ID:

Panel:

Gate: G5

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	SD	CV	Median	Peak	Peak Ch
All	1, 9647	6611	100.00	66.11	70.83	60.95	42.17	59.53	60.43	206	54
M1	100, 9647	1241	18.77	12.41	140.08	134.79	43.27	30.89	124.09	123	100

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